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Separon HEMA modified for immobilized metal ion affinity chromatographic separation of proteins

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ABSTRACT

Two sorbents for high-performance immobilized metal ion affinity chromatography were synthesized on the basis of Separon HEMA methacrylate matrices. Iminodiacetic acid was covalently bound on the surface of the sorbents by the means of two spacers of different length. In addition to the fact that both sorbents could be loaded with similar amounts of Cu^{2+} ions, their chromatographic properties were different, which was demonstrated by the separation of four globular proteins.

INTRODUCTION

Numerous proteins are able to form coordinate–covalent bonds with unoccupied coordination sites of metals via electron donor groups resident on their molecular surface. This ability was reported by Porath *et al.* [1] in the development of immobilized metal ion affinity chromatography (IMAC). In this method, the metal ion is immobilized on the surface of the sorbent via a chelating ligand. The ligand was iminodiacetic acid (IDA) covalently bonded to the expoxy-derived Sepharose [1]. This chelating ligand and reaction scheme have been used by most previous workers [2–9]. There are other methods for the synthesis of IDA carriers [10–13], but they are more complicated and usually do not provide more effective sorbents.

IDA has been the most frequent chelating ligand in IMAC; others have been used only rarely [2,11, 14]. On the other hand, the choice of immobilized metal ions is much wider. Cu^{2+}, Ni^{2+}, Fe^{3+} and especially Zn^{2+} have been used for the purification of proteins and peptides [2–4,6–8,13–26], but a number of other metals have also been examined [6,8]. Sorbents for IMAC have been synthesized on the basis of hydrophilic organic matrices [1,2,4–10] or silica gel [3,11–13]. In high-performance (HP) IMAC, silica gel or sufficiently rigid organic sorbents (Superose, TSK gel PW and some others) are used. Whereas the former offer better efficiency, the latter are much more hydrolytically stable and permit work even with alkaline buffered eluents.

Elution of adsorbed proteins is carried out using a pH gradient (sometimes increasing [21] but mainly decreasing [15–20,22,24–26]) or by increasing the concentration of amino acids, imidazole and ammonia in the eluent [2,4,22,23,27–29].

In addition to the separation and purification of proteins and peptides, IMAC can serve as a valuable tool in the study of their structure and topography [5,13,17,30]. In addition, IMAC sorbents can be used as regenerable carriers for immobilization of enzymes in biotechnology [31].

In this paper, we report the synthesis of two sorbents for HP-IMAC, based on Separon HEMA microparticulate hydrophilic methacrylate sorbents. The sorbents differed in the spacer arm length and in the hydrophilicity of the matrix.

The aim of this work was to confirm if the modification of Separon HEMA-BIO 1000 can provide a better matrix for IDA attachment then Separon HEMA 1000 E. In addition, we wished to verify whether the separation of proteins on the IMAC sorbents prepared could be influenced by

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ionic or hydrophobic interactions when the concentration of sodium chloride recommended in the literature was used.

EXPERIMENTAL

Sorbents

Separon HEMA 1000 E and HEMA-BIO 1000 (this sorbent was indicated as Separon HEMA 1000 H in a previous paper [32]) were purchased from Tessek (Prague, Czechoslovakia). Both sorbents were declared by the producer to have a molecular weight exclusion limit 10^6 and a wide pore-size distribution. The particle size is 10 μm.

Chemicals

Iminodiacetic acid (IDA) was obtained from Janssen Chimica (Beerse, Belgium), 1,4-butanediol diglycidyl ether (BUDGE) from Aldrich-Chemie (Steiheim, Germany), imidazole from Serva (Heidelberg, Germany) and ethylenediaminetetraacetic acid disodium salt (EDTA), NaCl, $Na_2HPO_4 \cdot H_2O$, and $CuSO_4 \cdot 5H_2O$ from Lachema (Brno, Czechoslovakia). Chicken egg white lysozyme (Lys), bovine pancreatic ribonuclease-A (RNase) and horse skeletal muscle myoglobin (Myo) were purchased from Sigma (St. Louis, MO, USA) and human transferrin (Tra) from Serva.

Chromatography

All experiments were performed with a Model 2150 high-performance liquid chromatographic pump, a low-pressure gradient mixer, a Model 2152 LC controller, a C6W-HC injector, a Model 2140 rapid spectral detector and a Data Print computer from Pharmacia–LKB (Bromma, Sweden). Sorbents were packed into stainless-steel columns (100 × 4 mm I.D.). A pressure of 2.5 MPa was maintained during the whole operation.

The loading of sorbents with copper was done by washing the column successively with 5 ml of 0.05 *M* EDTA, 10 ml of water, 7.5 ml of 0.1 *M* $CuSO_4$ solution and 10 ml of water. The flow-rate was 0.5 ml/min.

Synthesis of sorbents

Separon HEMA-BIO 1000–BUDGE. This epoxy-activated product was prepared by a slightly modified version of the method published by Porath *et al.* [1]. A 5-g amount of Separon HEMA-BIO 1000 was suspended in a mixture of 18 ml of 0.4 *M* NaOH, 18 ml of BUDGE and 25 mg of $NaBH_4$. The suspension was stirred for 8 h at 25°C, then washed with 300 ml of water and 50 ml of acetone and dried overnight at 35°C.

Separon HEMA 1000 E–IDA. Two reaction conditions were applied to bond IDA to the epoxy-activated sorbent Separon HEMA 1000 E.

Reaction scheme I is Porath's method [1]. A 4.5-g amount of IDA disodium salt and 80 mg of $NaBH_4$ were diluted in 83 ml of 2 *M* Na_2CO_3, then 5 g of Separon HEMA 1000 E were suspended in this solution and stirred for 8 h at 65°C. The suspension was subsequently kept at 65°C overnight, then the sorbent was filtered, washed with 0.1 *M* NaOH, water and acetone and dried for 6 h at 65°C.

In reaction scheme II, 8.7 g of IDA and 4.1 g of NaOH were diluted in 23 ml of water and the pH was adjusted to 10.0 with concentrated H_3PO_4. A 5-g amount of the matrix mentioned above was suspended in this solution and the suspension was kept at 65°C for 24 h. The suspension was shaken vigorously at 15-min periods for the first 8 h. Washing and drying were carried out as in reaction scheme I, then the sorbents were suspended in 100 ml of a 1 *M* solution of ethanolamine and kept for 8 h at 65°C with occasional shaking.

Separon HEMA-BIO 1000–Budge–IDA. This epoxy-activated matrix was treated by both reaction schemes described for Separon HEMA 1000 E.

Determination of content of epoxy groups in matrices

The method published by Pribyl [33] was used. A 0.2-g amount of sorbent was suspended in 20 ml of a 0.5 *M* solution of tetrabutylammonium bromide in glacial acetic acid and titrated with a 0.1 *M* solution of perchloric acid in glacial acetic acid. The equivalence point was determined potentiometrically.

Analysis of synthesized sorbents

The content of IDA in the prepared sorbents was determined by elemental analysis for nitrogen. Its loading capacity for copper was determined in the following way: 0.2–0.3 g of dry IDA sorbent was suspended in 25 ml of 0.05 *M* $CuSO_4 \cdot 5H_2O$ solution and mixed for 30 min. The sorbent was then filtered and washed with 100 ml of 0.1 *M* acetate buffer (pH 4.5) containing 0.5 *M* NaCl and with 50

ml of water. Cu^{2+} ions were then eluted with 100 ml of 0.05 *M* EDTA and measured spectrophotometrically at 735 nm.

RESULTS

Two synthesis routes, outlined in Fig. 1, were used for the preparation of IDA sorbents. The reaction of IDA with epoxy-activated matrices was carried out under strongly alkaline conditions with 2 *M* Na_2CO_3 (reaction scheme I) or at pH 10.0 (reaction scheme II). The results are summarized in Table I. The concentration of epoxy groups in Separon HEMA 1000 E (henceforth referred to as HEMA E) was 850 μmol/g of dry material (this value is declared by the producer) and Separon HEMA-BIO 1000–BUDGE (henceforth referred to as HEMA–BUDGE contained 520 μmol/g of glycidyl groups (this value was determined by the method described under Experimental).

The ability of both sorbents for aminolysis was tested by reaction with a large excess of a 1 *M* solution of ethanolamine (EA) at 65°C for 8 h. The molar content of nitrogen, equal to the proportion of converted epoxy groups, was determined by elemental analysis. A small increase in mass due to EA attachment was considered in the calculations.

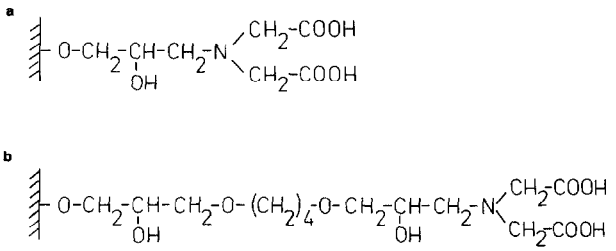

Fig. 1. Structures of (a) Separon HEMA 1000 E–IDA and (b) Separon HEMA-BIO 1000–BUDGE–IDA.

The average values for nitrogen in three parallel experiments were 660 μmol/g for HEMA E and 425 μmol/g for HEMA–BUDGE. It is probable that hydrolysis of epoxy groups proceeded simultaneously because no epoxy groups were determined after EA treatment. The hydrolysis of epoxy groups with 2 *M* Na_2CO_3 was also followed. No epoxy groups were determined after treatment for 24 at 65°C. The content of IDA was determined by elemental analysis for nitrogen in the same way as for EA.

Apart from the content of glycidyl groups, the main difference between the matrices discussed here is that these groups are attached directly to the

TABLE I

INFLUENCE OF MATRIX AND REACTION SCHEME ON SOME SORBENT PARAMETERS

No.	Sorbent[a]	Reaction scheme	Content of epoxy groups in matrix (μmol/g)	Content of N after reaction with IDA (μmol/g)	Content of N after reaction with EA[b] (μmol/g)	Content of Cu (μmol/g)
1	Separon HEMA 1000 E–IDA I	I	850	Below detection limit	Below detection limit	11
2a	Separon HEMA 1000 E–IDA II	II	850	332	610	135
2b	Separon HEMA 1000 E–IDA II	II	850	332	334	137
3	Separon HEMA-BIO 1000–BUDGE–IDA I	I	520	180	184	121
4	Separon HEMA-BIO 1000–BUDGE–IDA II	II	520	220	390	147

[a] All data are averages of two parallel experiments for HEMA–BUDGE and three parallel experiments for HEMA E.
[b] The treatment with ethanolamine was performed after the reaction with IDA.

surface in HEMA E whereas they are situated at the end of a relatively long spacer in HEMA–BUDGE.

It is interesting that IDA was bonded on HEMA–BUDGE under the conditions of reaction scheme I, whereas HEMA E did not bind any IDA if 2 *M* Na_2CO_3 was applied (see sorbents 1 and 3, Table I). The epoxy groups present in the matrices were hydrolysed during the reaction in 2 *M* Na_2CO_3. No reaction with EA was observed (see Table I).

When reaction scheme II was applied, almost the same proportion of epoxy groups was converted into IDA moieties (about 56%) in both matrices. The subsequent reaction with EA decomposed non-converted glycidyl groups. The final content of nitrogen (see sorbents 2 and 4, Table I) was lower then that obtained by the direct reaction with EA, probably owing to the hydrolysis proceeding during reaction with IDA. It seems that steric effects do not play a significant role in the conversion of epoxides into IDA groups under the given conditions. Even an extended reaction time (48 h) did not increase the degree of conversion with either sorbent.

On the other hand, steric effects can play a role in the loading of sorbents with Cu^{2+}. HEMA–BUDGE carrying IDA at the end of the spacer arm is capable of retaining a relatively larger amount of metal (67% of IDA groups) than HEMA E (42% of IDA groups), where the original glycidyl groups were attached directly to the surface of the matrix (see sorbents 2a, 3 and 4, Table I). Fleminger *et al.* [34] discussed a similar property of bonded epoxy groups.

The situation was different when reaction scheme I was applied. Whereas the content of IDA was only 18% lower in comparison with reaction scheme II when using HEMA–BUDGE, virtually no IDA was found after the reaction with HEMA E (see sorbent 1, Table I). The fact that no IDA was bound and no residual epoxy groups were present after the reaction can be explained only by the preferred hydrolysis of epoxy groups during the reaction with 2 *M* Na_2CO_3. The glycidyl groups attached directly to the surface of the matrix are probably less accessible for IDA than the same groups situated at the end of the spacer in HEMA–BUDGE and, in addition, the hydrolysis is much faster in 2 *M* Na_2CO_3 than at pH 10.0. Hence it is possible that virtually all epoxy groups of HEMA E were hydrolysed before they could be converted into IDA groups.

The question of whether EA bonded during the decomposition of residual epoxy groups could influence the loading of the sorbent with copper was also solved. The sorbent prepared according to reaction scheme II was treated partly with EA and partly with Na_2CO_3 after the reaction with IDA to decompose residual glycidyl groups. No difference was found in the loading capacity with copper (see sorbents 2a and 2b, Table I).

An additional difference between the two types of IDA sorbents is in the "effective" loading capacity. If the number of micromoles of IDA present on the sorbent is the limiting value for Cu^{2+} content, then HEMA E retained about 49% of this capacity (see sorbents 2a and 2b, Table I) whereas for HEMA–BUDGE this value was about 67% (see sorbents 3 and 4, Table I). This means that not all IDA ligands chelated Cu^{2+} ions with the same strength and some metal ions were washed out with acidic buffer or not all IDA ligands are accessible to Cu^{2+} ions [34].

To compare the abilities of the two sorbents in the chromatography of proteins, they were used for the separation of a model mixture of four globular proteins under the conditions optimized for each of the sorbents individually. Although the amount of Cu^{2+} chelated was nearly identical for both columns, substantial differences were observed. The resolution of proteins was much better on HEMA–BUDGE and they eluted as sharper peaks (see Figs. 2 and 3).

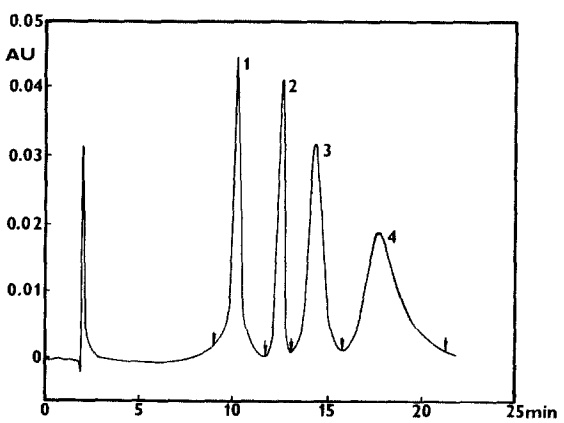

Fig. 2. Separation of model mixture on Separon HEMA 1000 E–IDA–Cu (see sorbent 2a in Table I). Column, 100 × 4 mm I.D.; flow-rate, 0.5 ml/min; gradient from 2 to 25 m*M* imidazole in 0.02 *M* Na_2HPO_4–0.5 *M* NaCl (pH 7.0) in 20 min. Proteins: 1 = Lys; 2 = RNase; 3 = Myo; 4 = Tra. A 50-μg amount of each protein was loaded. Absorbance was measured at 280 nm.

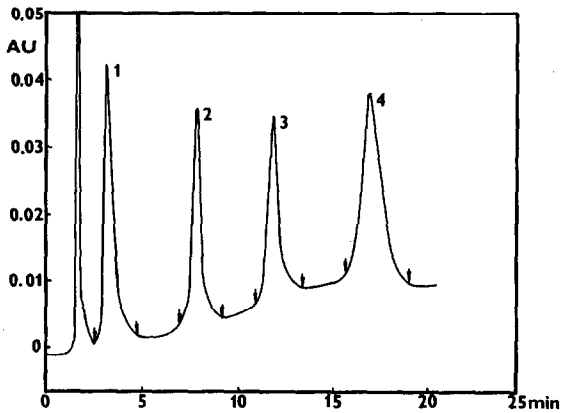

Fig. 3. Separation of model proteins on separon HEMA-BIO 1000–BUDGE–IDA–Cu (see sorbent 4 in Table I). For proteins and conditions, see Fig. 2.

In addition to the chromatographic resolution, the reproducibility of Cu^{2+} loading and its leakage during gradient elution are important factors concerning the routine application of this type of sorbent. The following experiments were made: columns were loaded with copper (see Experimental) and the gradient elution of the four above-mentioned proteins was performed. The volumes of effluent marked by arrows (see Figs. 2 and 3) were collected and the amounts of copper were determined. The columns were then washed with EDTA and water and repeatedly loaded with copper. The amount of copper loaded was also determined. The results of five repeated experiments are summarized in Table II. The loading reproducibility was better than 3% and copper leakage occurring with the concentration gradient of imidazole was negligible. Repeated gradient elution of the model mixture without washing out and new loading of copper shows that this very small loss of metal cannot influence the capacity factors of proteins. In these experiments we also measured spectrophotometrically the recovery of proteins and in all instances obtained values from 90% to 95%.

The following study was performed with HEMA–BUDGE only. It was mentioned above that part of the IDA moieties are free from Cu^{2+} and so their carboxyl groups can act as ion-exchanging groups. In the following experiments, the influence of the concentration of NaCl in the eluent was tested. The aim was to establish the contribution of ion-exchange effects to the retention of proteins. The results are given in Table III. The large shifts in the retention times of Lys and Tra obtained with 0.1 *M* NaCl can be explained in the following way. The electrostatic interaction of free carboxyl groups of IDA are not suppressed sufficiently with 0.1 *M* NaCl, so Lys (a protein positively charged and retained on cation exchangers at pH 7.0) was retained more with lower concentrations of NaCl. The opposite situation was observed with Tra (a protein negatively charged at pH 7.0), which is excluded by negatively charged carboxyl groups at pH 7.0 and in 0.1 *M* NaCl. The retention time of Lys decreased and that of Tra increased when the concentration of NaCl increased. The charges of two other proteins, Myo and RNase, are nearly

TABLE II

TEST OF Cu LEAKAGE DURING THE ELUTION OF PROTEINS WITH IMIDAZOLE GRADIENT

Exp. No.	Separon HEMA 1000 E–IDA–Cu					Separon HEMA-BIO 1000–BUDGE–IDA–Cu				
	Content of Cu in peak of protein (μg)				Content of Cu in column (μg)	Content of Cu in peak of protein (μg)				Content of Cu in column (μg)
	Lys	RNase	Myo	Tra		Lys	RNase	Myo	Tra	
1	0.2	0.1	0.2	0.5	3415	0.2	0.1	0.2	0.6	3581
2	0.2	0.1	0.2	0.5	3200	0.1	0.1	0.2	0.6	3645
3	0.2	0.1	0.2	0.5	3245	0.1	0.1	0.2	0.5	3511
4	0.5	0.2	0.8	1.8	3245	0.1	0.1	0.3	0.7	3397
5	0.3	0.2	0.5	1.5	3245	0.1	0.1	0.2	0.6	3664

TABLE III

INFLUENCE OF NaCl CONCENTRATION IN THE ELUENT ON PROTEIN RETENTION

Sorbent: Separon HEMA-BIO 1000–BUDGE-IDA-Cu. Gradient conditions: (A) 0.02 *M* phosphate buffer–NaCl as indicated–0.002 *M* imidazole (pH 7.0); (B) 0.02 *M* phosphate buffer–NaCl as indicated–0.025 *M* imidazole (pH 7.0); gradient from 0 to 100% B in 20 min.

NaCl concentration (mol/l)	t_R (min)			
	Lys	RNase	Myo	Tra
0.1	6.34	6.87	11.14	12.11
0.5	3.25	7.94	11.97	17.30

balanced at pH 7.0, so the change in the t_R values of these proteins was small when the concentration of NaCl was changed.

We also followed the contribution of hydrophobic interactions of proteins with the sorbent when a combination of an increasing gradient of imidazole and a decreasing gradient of $(NH_4)_2SO_4$ was applied. The results are summarized in Table IV. It is seen that the retention of all the proteins increased with increasing concentration of $(NH_4)_2SO_4$ in eluent A. This means that hydrophobic interactions play some role in the separation process, in addition to the interactions with immobilized metal, only at a high concentration of $(NH_4)_2SO_4$. This is also demonstrated by the last line in Table IV. In this instance the sorbent without immobilized Cu^{2+} was used. Because the hydrophobicity of the sorbent was low, a higher initial concentration was used to obtain comparable retention of solutes. Because the IMAC mechanism did not take part in the separation process in this instance, a change in retention order occurred and the most hydrophobic protein, Lys, was eluted as the last peak.

TABLE IV

INFLUENCE OF SIMULTANEOUSLY INCREASING IMIDAZOLE GRADIENT AND DECREASING $(NH_4)_2SO_4$ GRADIENT ON PROTEIN SEPARATION

Sorbent: HEMA-BIO 1000–BUDGE-IDA-Cu. Gradient conditions: (A) 0.02 *M* phosphate buffer–0.5 *M* NaCl–0.002 *M* imidazole–$(NH_4)_2SO_4$ as indicated (pH 7.0); (B) 0.02 *M* phosphate buffer–0.5 *M* NaCl–0.025 *M* imidazole (pH 7.0); gradient from 0 to 100% B in 20 min. For the last line: (A) 0.02 *M* phosphate buffer–0.5 *M* NaCl–3.0 *M* $(NH_4)_2SO_4$ (pH 7.0); (B) 0.02 *M* phosphate buffer–0.5 *M* NaCl (pH 7.0); gradient from 0 to 100% B in 20 min.

$(NH_4)_2SO_4$ concentration (mol/l)	t_R (min)			
	Lys	RNase	Myo	Tra
0	3.25	7.94	11.97	17.35
1.5	8.45	11.27	15.54	19.04
2.0	12.40	13.70	17.34	20.60
3.0	13.40	10.20	7.70	11.82

DISCUSSION

Two types of IDA–Separons were prepared. Whereas the reaction of IDA with epoxy-derived sorbents at pH 10.0 gave similar results, Porath *et al.*'s method [1] of synthesis (in 2 *M* Na_2CO_3) was successful only with the BUDGE-derived Separion where the epoxy groups are situated at the end of a long spacer arm. The steric hindrance of glycidyl groups attached directly to the surface of HEMA E probably plays a role in combination with the more rapid hydrolysis in 2 *M* Na_2CO_3, because no epoxy groups were left after the treatment in Na_2CO_3. Steric effects are not so important with HEMA–BUDGE and this sorbent gave similar results with both types of reaction. Steric effects can also affect the loading of the sorbent with Cu^{2+}. The "effective" loading capacity of HEMA–BUDGE was higher than that of HEMA E even if the content of IDA was higher in HEMA E.

The two types of sorbents show different chromatographic properties. Even if the chromatographic conditions were optimized for each type of sorbent, better resolution of the mixture of model proteins was observed on BUDGE-derived IDA–Separon. This improvement in resolution was not caused simply by narrowing of peaks, which was significant only with transferrin. The better resolution is a consequence of the favourable changes in the retention times of the model proteins. It seems that the spacer ligand affects not only the width of the peaks but also the mutual relationships between the retention times of proteins. The influence of ionic and hydrophobic interactions is negligible under the conditions of separation. A similar effect of the spacer was also observed with modified

Separon HEMA sorbents for hydrophobic interaction chromatography [32]. The introduction of a spacer between a matrix and a ligand probably reduces some undesirable interactions of proteins with the sorbent surface and positively influences the steric effects and mass transport during separation.

Synthesized Separon HEMA-BIO1000–BUDGE–IDA is a stable and rigid material. The elution of copper during separations is very low and the retention times of standards are reproducible even after 50 gradient cycles without new loading of the metal.

A paper dealing with practical applications of this sorbent is in preparation.

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